Published in final edited form as:

Prenat Diagn. 2017 December ; 37(13): 1364–1366. doi:10.1002/pd.5183.

Toxic metals in amniotic fluid and altered gene expression in cell-free fetal RNA

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Abstract

Both exposure to toxic metals, as well as deficiencies in essential metals, during pregnancy has been linked to a variety of negative reproductive outcomes. The exact etiologies of such outcomes and the effects of fetal exposure to these metals are largely unknown. Therefore, the ability to assess levels of these elements is critical to determining the underlying causes of such conditions and the effects that both essential and non-essential metals have in fetal development. Thus, using cell-free fetal RNA (cffRNA) from amniotic fluid, we set out to measure the association between amniotic fluid levels of toxic and essential metals and fetal gene expression. We find that arsenic was associated with increased expression of three genes known to play roles in both birth-related and reproductive effects. The results highlight the potential for detrimental health effects of prenatal metals exposure and the potential to identify biomarkers of environmental exposure during this critical developmental period.

Maternal exposure to toxic metals such as inorganic arsenic is associated with low birthweight, spontaneous abortion, stillbirth, and neonatal and infant mortality.^{1,2} In contrast, deficiencies in essential trace metals such as zinc and selenium are associated with pregnancy complications such as miscarriage and preeclampsia.³ The specific mechanisms by which toxic or essential metals affect pregnancy outcomes is largely unknown; however, metals are known to influence the expression of genes involved in critical biological pathways. Previous work has shown altered gene expression in newborns exposed to metals such as inorganic arsenic *in utero*⁴ and such alterations have been used to develop candidate biomarkers of disease, specifically in terms of preterm labor.⁵ Therefore, the ability to assess levels of these elements is critical to determining the underlying cause of such conditions and the effects that both toxic and essential metals have on fetal development. Thus, in a highly novel application with clinical relevance to prediction of obstetric complications, we

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Conflicts of interest: None declared

set out to measure the association between amniotic fluid levels of toxic and essential metals and fetal gene expression using cell-free fetal RNA (cffRNA) in a North Carolina cohort.

This pilot study leverages an existing retrospective case-control cohort examining spontaneous preterm birth (sPTB)⁶ and was approved by the institutional review board at the University of North Carolina at Chapel Hill. Briefly, eligibility criteria included women who delivered via TB or sPTB (defined as labor without a precipitating factor), with singleton fetuses without structural anomalies, and who underwent second-trimester (14-24 weeks) genetic amniocentesis performed for clinical indications. A total of 42 subjects (21 sPTB and 21 TB) were selected and matched by fetal sex, maternal age (within 2 years), gestational age (GA) at amniocentesis (within 1 week), race, year of amniocentesis (within 1 year), and medication exposure.

Metals were measured in amniotic fluid by Inductively-Coupled Plasma Mass Spectrometry (ICP-MS). This method uses a predefined instrument response curve (ion counts per unit concentration) to scan all masses from 7 to 238amu. Replicate samples were run and internal standards included with each sample to correct for matrix variations between samples.

Cell-free fetal RNA was extracted from amniotic fluid using the QIAamp Circulating Nucleic Acid extraction kit (Qiagen, Germantown, MD) according to the manufacturer's instructions but without the addition of carrier RNA. Cell-free fetal RNA was quantified using the Nanodrop 2000C (Thermo Fisher Scientific, Waltham, MA), and assessed for RNA integrity using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The cffRNA was hybridized to a custom made NanoString platform containing 65 genes of interest selected for their known biological roles in stress, inflammation, and oxidative stress, as well as 16 housekeeping genes. The NanoString digital barcoded technology allows for high precision, sensitive, direct multiplexed measurement of gene expression and is optimal for the small quantities of cffRNA found in amniotic fluid. Raw expression data was extracted using the nSolver software (NanoString Technologies, Inc, Seattle, WA) and normalized both to positive controls and housekeeping genes.

Associations between metals exposure and alterations in fetal gene expression were measured using multivariable regression analysis, controlling for maternal age, psychiatric medicines, race, fetal sex, and GA at delivery. A Grubbs test was used to identify outliers within the data set. Statistical analyses were performed using Partek Genomics Suite software (version 6.6; Partek Inc, St Louis, MO). Statistical significance was set at a false discovery rate (FDR)-corrected *p*-value of 0.05.

Demographic and exposure characteristics of the study population are summarized in Table 1. Using ICP-MS, we examined levels of three essential metals, selenium, manganese, and zinc, and three toxic metals arsenic, mercury and lead in amniotic fluid samples. Among the essential metals, the mean (range) amniotic fluid levels of selenium, manganese, and zinc were 9.7 ug/L (5.1-19.3 ug/L), 1.391 ug/L (0.164-3.8132 ug/L), and 71.7 ug/L (31.2-168.3 ug/L), respectively. The mean (range) amniotic fluid levels of arsenic, mercury, and lead were 16.3 ug/L (3.4 to 41.3 ug/L), 0.3769 ug/L (0.0811-0.8181 ug/L), and 0.280 ug/L (0.0258-1.9962 ug/L), respectively.

Multivariable models displayed that increasing arsenic levels in amniotic fluid were associated with the increased gene expression levels for three genes, namely Olfactory Receptor, Family 4, Subfamily S, Member 2 (*OR4S2*), Phospholipase C, Beta 1 (Phosphoinositide-Specific) (*PLCB1*), and Progesterone Receptor (*PGR*) (Figure 1). The identified genes are known to play roles in birth outcomes, reproductive effects, and in many cases to be responsive to toxic metal exposure. For example, *OR4S2* is a G-coupled protein receptor with similar domain structure to many neurotransmitter and hormone receptors. In terms of increased health risks, increased copy number variants (CNV) of *OR4S2* has previously been linked to increased risk of sPTB.⁷ *PLCB1* plays an important role in extracellular signaling and its upregulation has previously been associated with intrauterine growth restriction.⁸ With relevance to hormone signaling in pregnancy, *PGR* mediates physiological effects of progesterone. Of the genes, *PGR* has been previously associated with arsenic exposure where it has been shown that arsenic upregulates *PGR* protein expression, and increased expression levels are seen in female reproductive disorders.⁹ In addition to gene expression profiles, metals measured in amniotic fluid were also tested for their relationship to gestational age at delivery using a multi variable model with statistical significance set at FDR-corrected $p = 0.05$. None of the metals tested were associated with gestational age and only arsenic was associated with differential gene expression. These results were likely impacted by the pilot nature of the study and warrant replication in larger cohorts. Taken together, these data highlight that metal exposure early in life is associated with altered expression of critical target genes, and support prior work that showed that gene expression can be useful as a biomarker of toxic metal exposure.⁴

In summary, while toxic metals are known to cross the placental barrier and have been readily measured in cord blood serum at birth,¹⁰ amniotic fluid provides a unique biological matrix allowing for direct measure of both toxic metals exposure and alteration of fetal gene expression within the fetal compartment. Our findings show fetal exposure to heavy metals can be measured using amniotic fluid and that the NanoString technology can be applied to the measurement of fetal gene expression in this context. While maternal serum was not available for comparison within the present study, we demonstrated that fetal arsenic exposure is associated with differences in targeted gene expression in key pathways that may be related to spontaneous preterm birth. To the best of our knowledge, our study is among the first to measure the association between toxic metal exposure and fetal gene expression using a custom NanoString panel. Moreover, these results support the Developmental Origins of Health and Disease (DOHaD) hypothesis, highlighting the potential for detrimental health effects of prenatal toxic metals exposure and the need to identify biomarkers of environmental exposure during this critical developmental period. Future research will expand upon the current findings to examine the relationships between metals in amniotic fluid and infant health outcomes.

Acknowledgments

Funding sources: This research was supported by grants from the National Institute of Environmental Health Sciences (R01 ES019315, P42 ES005948, T32ES007018) and National Institute of Child Health and Human Development (R03 HD080788; 2K12HD00144116)

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What's already known about this topic?

- Prenatal toxic metal exposure has been linked to negative reproductive outcomes.
- Effects of fetal exposure to these metals on fetal gene expression is largely unknown.

What does this study add?

- This is the first study to show that *in utero* arsenic exposure may alter cell-free fetal RNA assessed in amniotic fluid.
- Differential fetal gene expression of *OR4S2*, *PLCB1*, and *PGR* is associated with amniotic fluid arsenic levels.

Figure 1. Arsenic-associated upregulation of *OR4S2*, *PLCB1*, and *PGR* with trend line and confidence intervals. Black data points denote ≥ 37 wks gestation, grey data points denote < 37 wks gestation.

Table 1

Demographic and exposure characteristics of study population.

Characteristics	Mean, Median, [Range] or N, (N%)
Maternal age (yrs)	35.1, 37 [17–44]
Psychiatric meds	11 (26)
Race	
Caucasian	25 (59)
African American	9 (21)
Hispanic	2 (5)
Asian	4 (10)
Unknown	2 (5)
Fetal Sex	
male	28 (67)
female	14 (33)
Gestational age at birth (weeks)	36.4, 37.3 [24.1–41]
Gestational age at amniocentesis (weeks)	17.6, 17.3 [14.7–23.3]
Selenium (ug/L)	9.7, 8.2 [5.1–19.3]
Manganese (ng/L)	1391, 1203.1 [164.9–3813.2]
Zinc (ug/L)	71.7, 61.9 [31.2–168.3]
Arsenic (ug/L)	16.3, 12.5 [3.4–41.3]
Mercury (ng/L)	36.4, 37.3 [24.1–41]
Lead (ng/L)	280.2, 157.5 [25.8–1966.2]